Universidade de Aveiro Departamento de Biologia
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COMPARISON OF DIFFERENT METHODS TO DETECT GENETIC BARRIERS IN A SMALL MAMMAL POPULATION

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica do Professor Doutor Carlos Fonseca, Professor Associado do Departamento de Biologia da Universidade de Aveiro e pela co-orientadora Doutora Clara Grilo, Investigadora no Departamento de Biologia da Universidade Federal de Lavras (Brasil).

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palavras-chave Fluxo genético, barreira genética, conectividade, genética de paisagem, rodentia, estradas, rios

resumo

A fragmentação do habitat e a consequente perda da conectividade entre populações pode reduzir o intercâmbio de indivíduos e consequentemente o fluxo genético, aumentando as hipóteses de ocorrer consanguinidade e consequentemente aumentar o risco de extinção local. A disciplina da genética da paisagem fornece cada vez mais e melhores ferramentas para detectar barreiras genéticas. No entanto, não se conhecem até à data, comparações de métodos em termos de consistência de resultados com dados observados e espécies com reduzida capacidade de dispersão. O objectivo deste estudo é avaliar a consistência dos resultados de cinco métodos de análise do papel da auto-estrada e de um rio como barreira ao fluxo genético numa população de rato-cego-mediterrânico *Microtus duodecimcostatus*: estimativas do F-estatistico, método de aglomeração não-Bayesianos, métodos de aglomeração Bayesianos, método de detecção de fronteiras (algoritmo Monmonier) e o teste Mantel simples e parcial. Todos os métodos testados foram consistentes em considerar o rio como uma não barreira genética ao rato-cego-mediterrânico. No entanto, não houve consistência nos resultados quanto ao papel da auto-estrada como barreira genética. As estimativas do F-estatistico, os métodos de aglomeração Bayesianos e o teste de Mantel parcial que mostram que a auto-estrada pode estar a funcionar como um filtro ao movimento dos indivíduos entre os dois lados da estrutura. Os métodos de deteção de fronteiras (algoritmo Monmonier) e de aglomeração não-Bayesiano não detectaram diferenciação genética nas populações de rato-cego-mediterrâneo devido à estrada. Com base nos nossos resultados nós recomendamos a aplicação dos testes de Mantel, os métodos de aglomeração Bayesianos e dos métodos de detecção de fronteiras para esclarecer o papel dos atributos da paisagem como barreiras genéticas uma vez que, todos foram capazes de detectar barreiras mas não obtiveram resultados similares. Apesar dos estudos com base em simulações apontarem as vantagens e desvantagens de cada método e os fatores que associados aos resultados, é necessário que se façam testes com base em dados reais para que sejam mais eficazes na detecção de barreiras genéticas.

keywords Gene flow, genetic barrier, connectivity, landscape genetics, rodentia, roads, streams

abstract Habitat fragmentation and the consequently the loss of connectivity between populations can reduce the individuals interchange and gene flow, increasing the chances of inbreeding, and the increase the risk of local extinction. Landscape genetics is providing more and better tools to identify genetic barriers.. To our knowledge, no comparison of methods in terms of consistency has been made with observed data and species with low dispersal ability. The aim of this study is to examine the consistency of the results of five methods to detect barriers to gene flow in a Mediterranean pine vole population *Microtus duodecimcostatus*: F-statistics estimations, Non-Bayesian clustering, Bayesian clustering, Boundary detection and Simple/Partial Mantel tests. All methods were consistent in detecting the stream as a non-genetic barrier. However, no consistency in results among the methods were found regarding the role of the highway as a genetic barrier. F*st*, Bayesian clustering assignment test and Partial Mantel test identifyed the highway as a filter to individual interchange. The Mantel tests were the most sensitive method. Boundary detection method (Monmonier's Algorithm) and Non-Bayesian approaches did not detect any genetic differentiation of the pine vole due to the highway. Based on our findings we recommend that the genetic barrier detection in low dispersal ability populations should be analyzed with multiple methods such as Mantel tests, Bayesian clustering approaches because they show more sensibility in those scenarios and with boundary detection methods by having the aim of detect drastic changes in a variable of interest between the closest individuals. Although simulation studies highlight the weaknesses and the strengths of each method and the factors that promote some results, tests with real data are needed to increase the effectiveness of genetic barrier detection.

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INTRODUCTION

1. INTRODUCTION

The major concern of conservation biologists is the loss of biodiversity which is mainly related with habitat loss, degradation and fragmentation (Cardinale et al., 2012; Forester and Machlist, 1996). The loss of habitat connectivity between the populations can reduce the individuals interchange and consequently gene flow (Coulon et al., 2004), increasing the chances of inbreeding, and consequently increase the risk of local extinction (Born et al., 2008; Epps et al., 2005; Gauffre et al., 2008; Keller & Largiadèr, 2003; Leidner & Haddad, 2010; McCulloch et al., 2013).

In humanized landscapes, species habitats comprise many features, like rivers, mountains, forests, grasslands and linear infrastructures (e.g. roads and railways) that in some cases can limit daily and dispersal movements affecting individual fitness and population viability in a long-term (Carmichael et al., 2001; Coulon et al., 2006; Evans et al., 2011; Geffen et al., 2004). Streams can act as a genetic barrier for several species that cannot move in the water (Storfer et al., 2007). For example, Mackenzie River in northern Canada seem to act as a genetic barrier for grey wolves (Carmichael et al., 2001) and in western England, the Wye River is limiting the movements of the European badger population, creating a significant genetic differentiation between populations (Frantz et al., 2010). Likewise, roads can also act as barrier or filter to movement of several species (Balkenhol & Waits, 2009; Redon et al., 2015). For instance, road has reduced genetic diversity of desert bighorn sheep in 15% in the last 40 years (Epps et al., 2005) and a major highway in central Germany was identify as a significant barrier to gene flow in a European wildcat population (Hartmann et al., 2013). On the other hand, several studies show that gene flow and genetic diversity are not affected by the presence of streams or roads. Rivers and Inland lochs of Scottish Highlands did not affect gene flow of red deer populations (Pérez-Espona et al., 2008). Also, in southwestern France, none of the studied landscape features (highway, rivers and several canals) were limiting the movements of the roe deer population (Coulon et al., 2004). These facts highlight the importance of studying the role of landscape features like streams and roads on the patterns of gene flow, to better understand the responses of species to these natural and artificial structures (Laurence et al., 2013; Manel et al., 2003; Prunier et al., 2013).

Landscape genetics is providing more and better tools to identify genetic barriers (Holderegger & Wagner, 2008; Manel et al., 2003; Storfer et al., 2007; Storfer et al., 2010). Methods such as Mantel's test, Wright's F-statistics (F*st*) or methods derived from Assignment tests are commonly used in a wide range of studies for the detection of

genetic barriers (Buchalski et al., 2015; Hartmann et al., 2013; Laurence et al., 2013; Šprem et al., 2013; Vergara et al., 2015). However, these methods are not consistent in detecting genetic barriers. To detect if a highway was acting as a barrier to movement on two species, bank vole *Myodes glareolus* and yellow-naked mouse *Apodemus flavicollis*, Rico et al. (2009) found that F*st* only shows significant differentiation between one pair of populations of bank voles, and the non-spatial Bayesian clustering analysis identified two different groups on both species not related to the highway. In another study with common vole, *Microtus arvalis* all three analysis show different results, the non-spatial Bayesian method show the presence of just one cluster, and spatial Bayesian approaches show two and four different clusters, Geneland and Tess respectively (Gauffre et al., 2008). Yet, the authors conclude that unexpectedly, the motorway considered as a likely barrier to dispersal was not associated with any spatial genetic discontinuity. Because there is some inconsistency among the methods several studies performed simulations to evaluate the effectiveness of those methods. The spatial Bayesian clustering methods seem to be the best available tools for detecting linear genetic barriers (Blair et al., 2012; Safner et al., 2011) whereas Non-Bayesian clustering method (DAPC) proved to be insufficient to detect fine-scale dispersal patterns (Jones & Wang, 2012). With DAPC, barrier was only detected when dispersal distance was above 60km (e.g., Blair et al., 2012). Simulations also show that probability of site occupancy and population size seem to be key factors in determining the spatial genetic structure of population (Epperson et al., 2010). Prunier et al., (2013) also detected some inconsistency in results regarding the role of the highway as a genetic barrier when run real and simulated data for alpine newt *Ichthyosaura alpestris*. In this study, the simulation suggests that the roads were a significant barrier to gene flow whereas the empirical genetic data did not support those results. Furthermore, these studies performed comparisons among methods in terms of effectiveness but to our knowledge no comparison of methods regarding consistency has been made with observed data and species with low dispersal ability.

The aim of this study is to examine the consistency of the results and accurate which is the most accurate approach of five different available tools to detect barriers to gene flow in a natural population of small mammal species. We used a Mediterranean pine vole *Microtus duodecimcostatus* population as a model to detect genetic changes due to a stream and a highway built 20 years ago. The pine vole is a fossorial rodent (Duarte et al., 2015), common in central Europe (Santos et al., 2010). Although, pine voles are habitat generalists, they need small burrows for refuge and food (Briner et al., 2005) and suffer variations due to habitat quality and climate (Paradis, 1993). Pine vole is monogamous

and females can breed in groups or alone, which influences the age at maturity and in case of solitary females, they reach sexual maturity earlier, which increases their reproductive success (Boyce & Boyce, 1988; Paradis, 1993) and their dispersal ability is around 200m (unpublished data).

Since the majority of previous studies used species with minimum dispersal distance of more than 10km (Blair et al., 2012; Landguth et al., 2010), we aim to add crucial information on the detection of genetic barriers in populations with very low dispersal range. We expect consistency in the Bayesian approaches, although they consider different information about the population, by grouping the individuals in a way to minimize the instability of the connections. Wright's F-statistics and Partial Mantel test may show similar results because in species with low dispersal ability they both show identical results (e.g. Landguth et al., 2010). We also expect a reliably relationship between Boundary detection method and the potential barriers because these in previous studies they high efficiency in detecting barriers for low dispersal species (Blair et al., 2012).

2. METHODS

2.1. Study Area

Mediterranean pine vole samples were collected in southern Spain between Huelva and Seville (Figure 1). Altitude ranges from 100 to 140 m above sea level. It is defined by a Mediterranean climate, with hot and dry summers and unstable wet winters. The land is mostly used for intensive agriculture, mainly olive tree plantations, which allow for the existence of small remnants of non-tilled pasture around the trees, interpatched with vineyards and cereal crops. The area is crossed by one 4-lane highway (A49) with an average width of 27m and a median section covered with natural shrubby vegetation. The average daily traffic intensity in 2011 was 31 842 vehicles for the highway (source: Ministerio de Fomento, España and Consejeria de Fomento y Vivienda, Junta de Andalucía). In the study area a stream is present and cross the highway, which splits the set of samples in three groups (Figure 1).

2.2. Sample collection

Mediterranean pine voles (n=165) were live trapped during the one-year period in the study area (Figure 1). Samples were collected in three groups: on both sides of the highway (north – A49N and south – A49S) and on both sides of the stream at the north of the highway (west – SW and east – A49N). Animal trapping and handling was done according to the procedures of the Animal Care Committee of Junta Andalusia. Tail tip removal was the method for obtaining tissue samples for DNA analysis. All samples were preserved in 90% ethanol and stored at -20 °C.

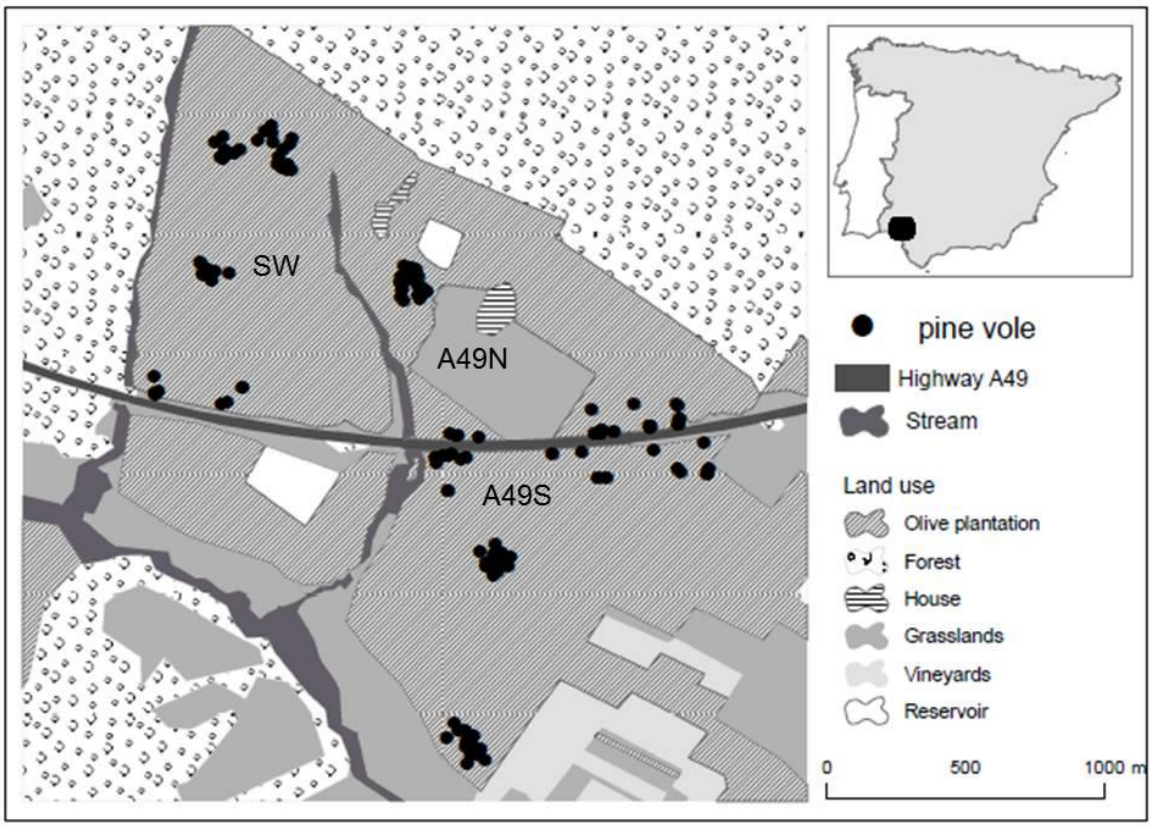

Figure 1 – Samples of Mediterranean pine vole in the study area (SW – all samples in the western part of the stream, A49N – all samples at the north part of the highway 49 and the east part of the stream and A49S – all samples in the southern of highway A49).

2.3. DNA Extraction and Microsatellites analysis

Total DNA isolation from all tissue samples was performed using a modified salt-extraction method (Bruford et al.1992). Mediterranean pine vole samples were genotyped with eleven microsatellite markers: Mar76, Mar12, Mar63, Mag21, MSMM2, Ma88, Mar3, Ma9, Mag13, Ma68, Mag18 (Jaarola et al.2007; Gauffre et al.2007; Ishibashi et al.1999; Ruda et al.2008).

Multiplex and single DNA amplification of all microsatellites was carried out in a 20ul volume containing 50-100ng template DNA, primers (0.2uM each), MgCl2 (2.0-2.5 mM), dNTPs (0.2mM) and 1.5u AmpliTaq Gold polymerase in 1X PCR buffer (Applied Biosystems, Foster City, CA). The amplification was started with a denaturation step at 95°C for 10 minutes, followed by 35 cycles (95°C for 50 seconds, 52-57°C for 60 seconds, 72°C for 50seconds), and an extension step at 72°C for 10 minutes. All reactions included

a negative control. Products were analysed on an ABI 3730 automated capillary sequencer and alleles were scored with GeneMapper v.4.0 (Applied Biosystems, FosterCity, CA, USA).

2.4. General Description of the methods

We evaluate the following five groups of methods to detect genetic barriers. These methods are described, summarized and examples of studies and species are given:

1. Wright's F*st* is a measure that describe the distribution of genetic variation between populations (Jones & Wang, 2012; Landguth et al., 2010; WRIGHT, 1949). Several studies used F*st* estimations for determining genetic differentiation between populations which indicate the roads, urban areas, open fields as possible barriers to gene flow in species like moor frog *Rana arvalis, common frog Rana temporaria,* Alpine newt *Triturus alpestris* and *wood frog Lithobates sylvaticus* (Arens et al., 2007; Buskirk, 2012; Gabrielsen et al., 2013).

2. Non-Bayesian clustering method (Discriminant Analysis of Principal Components - DAPC) is a multivariate method that is able to identify and characterize clusters of individuals (Buchalski et al., 2015; Federman et al., 2014; Jombart et al., 2010) assigning each individual to a pre-defined set of populations (Blair et al., 2012). Some studies used this tool to infer barrier effect of roads, streams, and other features of the landscape in species like Western ground snake *Sonora semiannulata* and stone marten *Martes foina* (Cox & Chippindale, 2014; Vergara et al., 2015).

3. The Bayesian clustering assignment tests assume that the closest individuals are more likely to belong to the same population and estimate the likely number of populations/subpopulations of the sample. This method can be tested in programs that do not use spatial information of individuals or in programs that included the spatial information of individuals (Chen et al., 2007). Several studies implemented this method for test the potential of roads, streams, lakes and several other features of the landscape as barriers in species like *Rana arvalis, rattlesnake Crotalus horridus, wood frog Rana sylvatica* and mountain pygmy possum *Burramys parvus* (Arens et al., 2007; Clark et al., 2010; Crosby et al., 2008; Mitrovski et al., 2007).

4. Boundary detection methods are able to identify transitional areas through the analysis of the individual genotypes (Safner et al., 2011). This method is based on Monmonier's algorithm, which calculates genetic distances between individuals over a connected network and among nearest neighbors (Blair et al., 2012). Some studies implemented Monmonier's algorithm to detect the effect of roads and large rivers to isolate the populations such as common midwife toad *Alytes obstetricans* and *stone marten* (Garcia-Gonzalez et al., 2012; Vergara et al., 2015)

5. Mantel's test method was applied in order to verify the presence of an isolation-by-distance (IBD) between groups of individuals or populations (Manel et al., 2003). Firstly, we can test for isolation by distance to verify if the distance between the groups is significant and if it's the only responsible for the isolation of the populations. Then, to verify the presence of a genetic barrier we can use Partial Mantel's test to estimate the correlation between three variables: genetic distance, geographic distance and presence of the barrier (Manel et al., 2003). Several studies used Partial Mantel test to detect the barrier effect of linear infrastructures and natural features of the landscape in species like bank vole *Clethrionomys glareolus* (Gerlach & Musolf, 2000) and muskrat *Ondatra zibethicus* (Laurence et al., 2013).

2.5. Data Analysis

We calculated the F*st* to examine genetic differentiation between the tree groups of individuals of both sides of the highway and both sides of the river using Arlequin 3.01 software (Excoffier et al., 2005) under 10 000 permutations.

We performed the non-Bayesian clustering analysis DAPC in R package ADEGENET (Jombart et al., 2010) that does not assume Hardy–Weinberg or linkage equilibrium (Federman et al., 2014; Jombart et al., 2010). We use ''find.cluster'' function for inferring the optimal number of clusters using the Bayesian information criterion (BIC). Then we implemented DAPC to characterize the clusters and choose to retain 20 Principal Components and all eigenvalues.

We run the Bayesian clustering methods using the STRUCTURE 2.3.4 and Tess3 softwares. STRUCTURE only uses genotype information and in Tess3 uses genotype and location of the individuals (Caye et al., 2015). In STRUCTURE we performed 10

independent runs for K from 1 to 8 carried out with 300 000 Markov chain Monte Carlo (MCMC) iterations and a burn-in of 10 000 iterations. The program run under admixture and correlated allele frequencies, with and without a priori information on geographic populations. The most likely value for K was estimated following the Evanno method (Evanno et al., 2005) implemented in STRUCTURE HARVESTER (Earl et al., 2012).

To run the Tess3 (Caye et al., 2015) all the parameters are performed simultaneously by the Markov chain Monte Carlo (MCMC) algorithm (Guillot et al., 2005). Firstly, we infer K setting to range between 1 and 8 and we run 10 independent repetitions for each k, with admixture using the CAR model, under 200 000 MCMC iterations and with 40 000 of burn-in. The modal cluster membership for each individual was selected and plotted on a map.

We also performed one boundary detection method, Monmonier Algorithm, using the Alleles in Space 1.0 software (Miller, 2005). We set the number of barriers to one, two and three to identify the closest individuals with more differences and where are the boundaries in those differences.

Simple and partial Mantel test analyses were performed including three environmental variables: genetic distance, geographic distance and presence of barrier (stream or highway). We run this analysis separately: we compare two groups bisected by the stream (SE and SW) and the two groups bisected by the highway (A49N and A49S). SPAGeDi v.1.2 software was used to calculate genetic distance among all pairs of individuals (Hardy & Vekemans, 2002) and GenAlex v.6.1 software was used to estimate geographic distance (Peakall & Smouse, 2006). The barrier model consists in a matrix which assigns a number, 1 or 0 to each individual as they are in the same side of the barrier or in the contrary side. We needed two barrier matrices for testing the stream and the highway. Initially, we started to test isolation by distance over Mantel test in R package ECODIST, which estimate the correlation between the genetic distance and geographic distance. Then we analyzed the effect of the stream through the correlation of genetic distance and stream matrix. We also analyzed the effect of the highway through the correlation of genetic distance and the highway matrix. To evaluate if the effect of the river and the highway was significant when distance effect was controlled (geographic distance partialled out) we performed a partial Mantel test in the same R package.

3. RESULTS

Mediterranean pine voles bisected by the highway (A49N and A49S) show significant genetic differentiation through F*st* test (F*st* = 0.0199; p-value<0.0005). The group bisected by the stream (SW and A49N) exhibit also significant genetic differentiation through F*st* (F*st* = 0.009; p-value=0.002) but with a low weight.

The Non-Bayesian clustering analysis shows three clusters (Figure 2a). However, these groups are not related with potential linear barrier such as highway or the stream (Figure 2b).

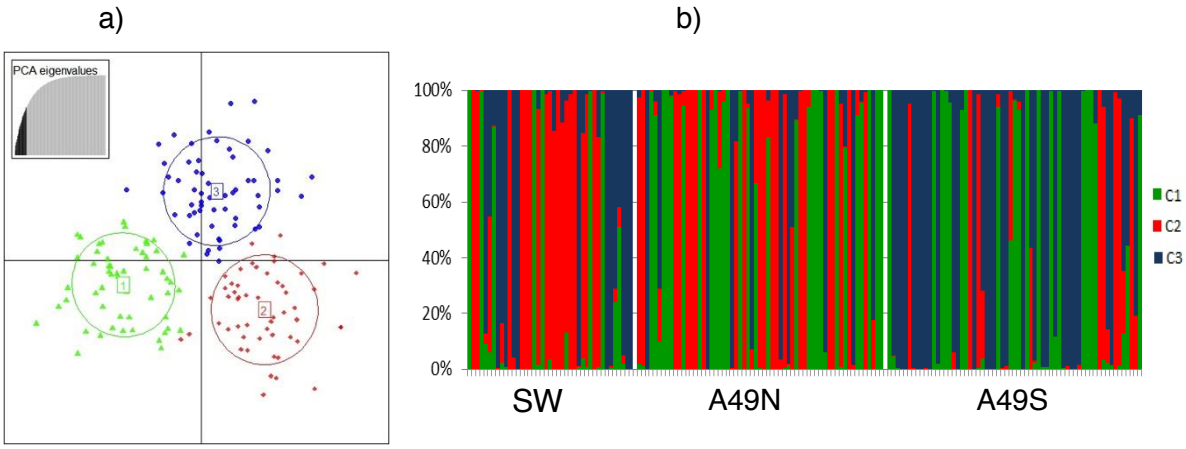

Figure 2 –Three clusters identified by the non-Bayesian clustering method (DAPC). a) Scatterplot and b) barplot showing the probability of population membership for each sampled individual. At the bottom of the barplot is sample location of the three groups of individuals (SW; A49N and A49S). Each colour corresponds to a different cluster.

The non-spatial Bayesian clustering assignment analysis using Structure software, inferred three as the optimal number of clusters. Results from Evano method indicated the presence of three different clusters. However, the probability of individuals to belong to the third cluster was lower than 0.6 and additional runs suggested that k=2 was a better model. After running Structure setting k=2 we see that this method show the association between the defined cluster and the presence of the highway (Figure 3).

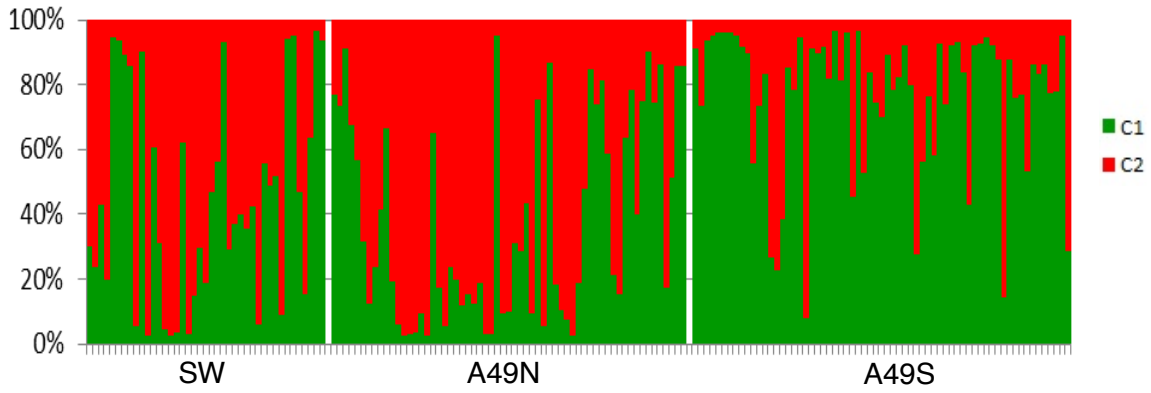

Figure 3 – Results from non-spatial Bayesian clustering assignment analysis (Structure software), setting k=2. Barplot showing the probability of population membership for each sampled individual and below the barplot are sample location of the two groups of individuals, north and south of the highway (A49N and A49S) and west side of the river (SW).

Spatial Bayesian clustering analysis, through Tess, showed a stabilized DIC with k=3 but, like in non-spatial Bayesian clustering analysis the third cluster was not significant. Figure 4 show the results for two different clusters.

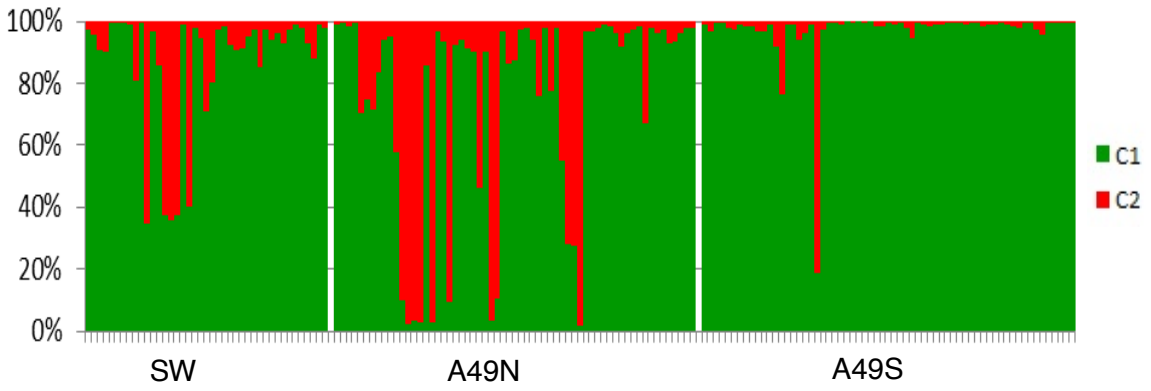

Figure 4 – Results from non-spatial Bayesian clustering analysis setting k=2. Barplot showing the probability of population membership for each sampled individual and below the barplot are sample location of the tree groups of individuals, north and south of the highway and west side of the river (in green is described the cluster 1, red the cluster 2).

Through the Boundary detection method (Monmonier algorithm) we set the number of barriers equal to one, two and three, consecutively. The first barrier corresponds to the group more different in their neighbourhood, the following identified barriers present fewer differentiation. Among the three identified barriers (Figure 5), we cannot relate none of them with the structures in study. In figure 5 are represented the location of the three identified barriers relatively with samples location with the stream and highway.

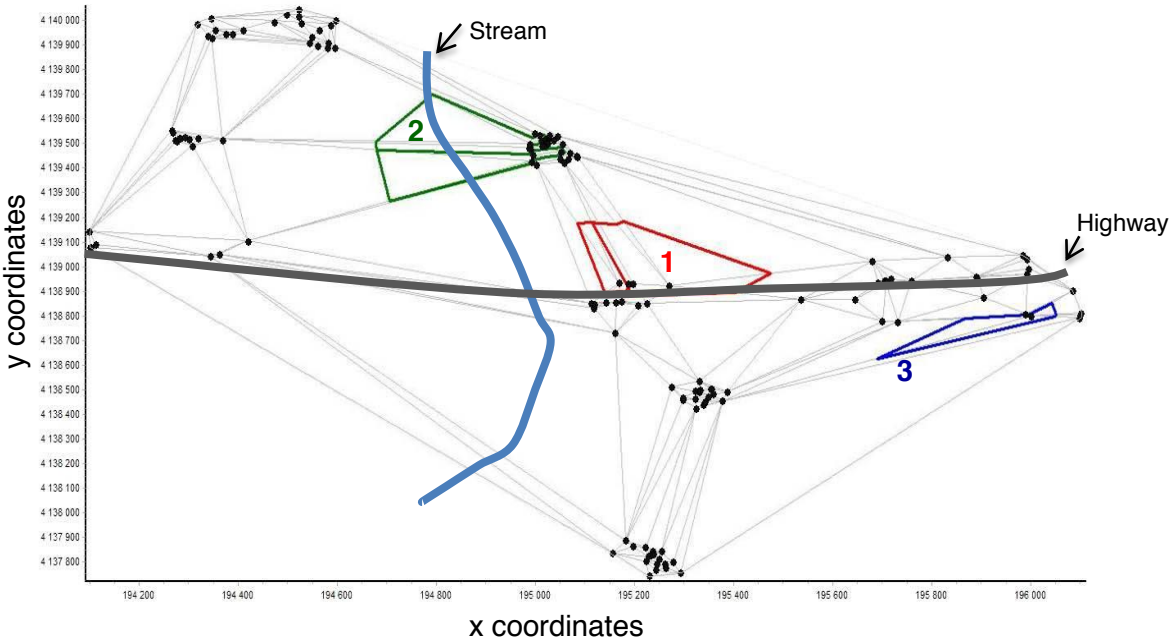

Figure 5 – Results from Boundary detection analysis setting the number of barriers to three. The boundaries of the three barriers are represented with different colours, and their order of detection is represented with the numbers 1, 2 and 3. The black points correspond to sample location sites, in dark grey are represented the highway and in blue are represented the stream. Coordinates projection 25830: ETRS89 / UTM zone 30N.

Finally, simple Mantel test analysis show a positive correlation between the geographic distance and genotypes of samples indicating a significant effect of IBD (Table 1). Partial Mantel test show a positive correlation between the genotypes, the geographic distance and the highway matrix suggesting that beyond the IBD effect the highway is also acting as a genetic barrier. However, Partial Mantel does not detect that the stream is acting as barrier (Table 1).

Table 1 – Simple and Partial Mantel tests for the two different structures (stream and highway). Site, N – number of individuals sampled; significance: *<0.05; **<0.005.

	Site	**N**	**Simple Mantel test – IBD**	**Partial Mantel Test** (controlling Euclidean distance)
Stream	SW A49N	41 61	0.085*	-0.019
Highway	A49N A49S	61 63	0.107**	0.047**

4. DISCUSSION

Genetic barriers usually occurs because of human activities such as new roads, agricultural exploitation and deforestation (Storfer et al., 2007). However, natural features of the landscape can also be a barrier to movement (Anderson et al., 2015). Understanding the role of natural and non-natural landscape features on species with limited ability to disperse is crucial to define strategies to mitigate the barrier effects.

Our findings reveal that all methods show that the stream is not a barrier for gene flow while for the highway there was no consistency among the methods. F*st*, Partial Mantel test and the Bayesian clustering methods suggest some genetic differentiation between the two groups bisected by the highway. The non-Bayesian clustering analysis defined three clusters without any relationship with the highway while the Boundary method (Monmonier's algorithm) did not considered the highway as a barrier.

Although F*st* estimates seem to indicate differentiation between the two groups of samples bisected by the highway, this information is not enough to confirm the presence of a genetic barrier. The significant differences obtained with this test suggest some disturbances in gene flow. There are several studies that use Fst estimations relating high differentiation between populations with the presence of a genetic barrier. For instance, Gerlach & Musolf (2000) found significant differentiation between bank vole populations bisected by a 25 year- old highway. In fact, Landguth et al., (2010) show that diferentiation through Fst test is only detected when the feature is acting as a barrier for a long time, at least 100 generations which correspond to 20 years of barrier presence if this species has in average five generations per year like the pine vole (Paradis, 1993). Thus, the detection of a weak genetic differentiation due to A-49 highway has two possible explanations: 1) this survey occurred 20 years after the highway construction finished and the barrier effect is only shown now; or 2) the highway is permeable to some individuals and is not acting as a barrier.

Likewise, Simple and Partial Mantel test show a weak but significant genetic differentiation due to highway. Despite having been the subject of much criticism (e.g. Manel and Holderegger, 2013) many recent studies have been using simple Mantel test to detect the effect of the distance on isolating populations (Berkman et al., 2013; Crosby et al., 2008; Kuehn et al., 2007; Landguth et al., 2010; Prunier et al., 2013; Vergara et al., 2015). Actually, we found that the simple Mantel test detected isolation by distance between the groups bisected by the stream and the groups bisected by the highway. In low dispersal ability species is expected that IBD is more pronounced (Blair et al., 2012).

Furthermore, our research was done in a heterogeneous landscape with vineyards, olive plantations and grasslands which intensify the the effect of distance on populations (Rico et al., 2009). Besides the IBD effect, Partial Mantel test detected the effect of the highway as a genetic barrier/filter. This test allows us to compare the effects of several variables and control the effect of distance to detect the barrier. Because Partial Mantel approach allows us to test different landscape scenarios, this test may assess in an accurate approach the potential barrier effect of landscape features. It is also known that partial Mantel test was able to detect recent barrier than the other methods (Landguth et al., 2010), which makes this method more interesting in detecting genetic barriers when the age of the barrier is not clear.

Both spatial and non-spatial Bayesian approaches showed consistency in the results indicating the highway may act as a filter to individual interchange. Although we can see differences among the two sides of the highway, there is no complete correspondence with the highway. Bayesian clustering assignment tests may be detecting preliminary genetic differences between the populations separated by the highway, which suggest that the process of differentiation due to the highway is in the beginning.

In contrast with the above tests, non-Bayesian clustering method DAPC was not able to reliably define genetic differentiation. The clusters identified did not match with the tested highway. In fact, other authors showed some difficulties to define clusters with DAPC for stone marten populations distributed for all Iberian Peninsula that correspond to potential barriers (Vergara et al., 2015).In contrast, Buchalski et al., (2015) were capable to describe three geographical areas through DAPC limited by roads and highway between for bighorn sheep (*Ovis canadensis nelsoni*), but further research is needed to confirm this finding. The efficiency of DAPC seems to increase with the increasing of distance dispersal of the species (Blair et al., 2012). Thus, non-Bayesian clustering method DAPC seem to show a lack of sensitivity to detect barriers and may not be appropriate for species with the low dispersal ability like Mediterranean pine voles or stone martens.

Contrary to our expectations, Boundary detection was not able to match the identified potential barriers with the highway. The Boundary detection method was already able to relate the road density with the increase of genetic distance between populations of common midwife toad (Garcia-Gonzalez et al., 2012). However, on effect of roads were found for the other amphibian species (palmate newt *Lissotriton helveticus*), which indicate that method can detect barriers and the species specific features may explain differences in the response towards the road (Garcia-Gonzalez et al., 2012).

The performance of each method depends on the time of barrier construction, on landscape composition and also on individual traits like size, dispersal ability, reproduction rate and behaviour (Blair et al., 2012; Jones & Wang, 2012; Landguth et al., 2010; Safner et al., 2011). As stated before, different methods applied to the same dataset can led to different results suggesting that the evaluation of the effectiveness and reliability of different statistical methods is crucial (Balkenhol et al., 2009). The consistency between Wright's F-statistics and Partial Mantel test may be due the date of the highway construction. Both seem to be sensitive to detect a barrier acting at least 100 generations. Bayesian clustering approaches show consistency on the obtained results because they all work at individual level and try to group the samples in study so that Hardy-Weinberg and the connections are minimized. The difference between them is the information included: the spatial Bayesian needs the location of the samples and the other do not consider the location of the samples. Non-Bayesian clustering methods and boundary detection method have different approaches: the non-Bayesian clustering defined the groups in by maximizing the variation between them, while boundary detection methods focus on the biggest genetic distance between the closest individuals.

Our results are not in line with Blair et al.(2012) who show that that Bayesian clustering methods are the best approach for all the studied scenarios. The lack of consistency between our work and Blair et al.(2012) may due to differences in dispersal ability, which in our study was very low.

Studying the patterns of gene flow is very important, because they can enable us of a better understanding of the factors allowing the identification of barriers to some species (Laurence et al., 2013; Manel et al., 2003). However, the results of some methods should be analysed with caution, since they can produce a high diversity results (Storfer et al., 2010). We were not able to evaluate the effectiveness of the methods because we do not know the role of the stream and the highway on the genetic differentiation of the pine vole. Based on these results, we recommend the application of Mantel test, Bayesian clustering and Boundary methods to clarify the role of landscape features as genetic barriers since all are able to detect barriers but do not reach to similar results. Although simulation studies highlight the weaknesses and the strengths of each method and the factors that promote some results, tests with real data are needed to increase the effectiveness of genetic barrier detection.

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5. REFERENCES

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